

# Diadenosine 5', 5'''- $P^1, P^4$ -tetrphosphate ( $Ap_4A$ ) is synthesized in response to DNA damage and inhibits the initiation of DNA replication

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## ABSTRACT

The level of intracellular diadenosine 5', 5'''- $P^1, P^4$ -tetrphosphate (Ap<sub>4</sub>A) increases several fold in mammalian cells treated with non-cytotoxic doses of interstrand DNA-crosslinking agents such as mitomycin C. It is also increased in cells lacking DNA repair proteins including XRCC1, PARP1, APTX and FANCG, while >50-fold increases (up to around 25  $\mu$ M) are achieved in repair mutants exposed to mitomycin C. Part of this induced Ap<sub>4</sub>A is converted into novel derivatives, identified as mono- and di-ADP-ribosylated Ap<sub>4</sub>A. Gene knockout experiments suggest that DNA ligase III is primarily responsible for the synthesis of damage-induced Ap<sub>4</sub>A and that PARP1 and PARP2 can both catalyze its ADP-ribosylation. Degradative proteins such as aprataxin may also contribute to the increase. Using a cell-free replication system, Ap<sub>4</sub>A was found to cause a marked inhibition of the initiation of DNA replicons, while elongation was unaffected. Maximum inhibition of 70-80% was achieved with 20  $\mu$ M Ap<sub>4</sub>A. Ap<sub>3</sub>A, Ap<sub>5</sub>A, Gp<sub>4</sub>G and ADP-ribosylated Ap<sub>4</sub>A were without effect. It is proposed that Ap<sub>4</sub>A acts as an important inducible ligand in the DNA damage response to prevent the replication of damaged DNA.

**Keywords:** Diadenosine tetrphosphate; DNA damage; DNA replication; ADP-ribosylation; cell signaling

<sup>1</sup>**Abbreviations:** Ap<sub>3</sub>A, diadenosine 5', 5'''- $P^1, P^3$ -triphosphate; Ap<sub>4</sub>A, diadenosine 5', 5'''- $P^1, P^4$ -tetrphosphate; Ap<sub>5</sub>A, diadenosine 5', 5'''- $P^1, P^5$ -pentaphosphate; Gp<sub>4</sub>G, diguanosine 5', 5'''- $P^1, P^4$ -tetrphosphate; ADPR-Ap<sub>4</sub>A, ADP-ribosylated diadenosine 5', 5'''- $P^1, P^4$ -tetrphosphate; MEF, mouse embryo fibroblast; MMC, mitomycin C; APTX, aprataxin; PARP, poly(ADP)ribose polymerase; SSB, single-strand break; XRCC1, X-ray repair cross-complementing protein 1; ARS, aminoacyl-tRNA synthetase

## 1. Introduction

The dinucleotide diadenosine-5',5'''- $P^1, P^4$ -tetrphosphate ( $Ap_4A^1$ , Fig. 1a) is synthesized in prokaryotes and eukaryotes in response to a variety of cellular stresses and stimuli and has been described as a pleiotropically acting alarmone with a role in establishing the response to these stresses, although few molecular details of its actions have ever been established [1-4]. Thus,  $Ap_4A$  has been implicated in the heat shock and DNA damage responses [5-7], apoptosis [8, 9] and in several signalling pathways [10-13].

$Ap_4A$  can be synthesized *in vitro* by several enzymes including aminoacyl-tRNA synthetases, firefly luciferase, DNA and RNA ligases, acyl-CoA synthetases and other ligases [13-15]. Of these, it is generally accepted that the aminoacyl-tRNA synthetases are primarily responsible for the steady-state level of  $Ap_4A$  *in vivo* [16]; however, the precise synthetic source of stress-induced increases may depend on the stimulus in question. In eukaryotes, the principal enzyme responsible for  $Ap_4A$  degradation is the NUDT2 nudix hydrolase [17, 18] although two HIT family proteins, the Fhit tumour suppressor [19] and aprataxin (APTX) [20], are also able to hydrolyse  $Ap_4A$  to some extent.

With regard to the DNA damage response, evidence gathered several years ago led to the hypothesis that DNA damage-induced  $Ap_4A$  had some role in regulating DNA replication in response to the damage [2-4, 21]. This evidence included the increase in  $Ap_4A$  observed after exposure of cells to DNA damaging agents, particularly those that cause single-strand breaks (SSBs) [5, 6, 22] and the ability of  $Ap_4A$  to bind specifically to a protein (still unidentified) associated with a multiprotein complex that included DNA polymerase- $\alpha$ /primase [23-25]. Detractors of this notion pointed to the lack of cell survival data associated with stress induction and suggested that only unphysiological, lethal stresses caused a significant increase in  $Ap_4A$  through a derangement of normal metabolism [26, 27]. This, plus the later recognition of the importance of PI3-type kinases in regulating DNA replication after DNA damage [28] led to a loss of interest in  $Ap_4A$  as a possible contributor to replication control. However, the recently recognized ability of small, highly phosphorylated molecules such as ppGpp (in bacteria) [29, 30] and inositol pyrophosphates (in mammalian cells) [31, 32] to directly regulate aspects of DNA replication and repair suggest that a re-examination of the role of  $Ap_4A$  in these processes is warranted. We now show here that non-cytotoxic doses of certain DNA damaging agents increase  $Ap_4A$  to concentrations that can inhibit the initiation of DNA replication in a mammalian cell-free system and provide some pointers to the mechanism underlying this increase and its function. In addition, we demonstrate the accumulation *in vivo* of previously undetected ADP-ribosylated derivatives of  $Ap_4A$  in response to DNA damage.

## 2. Materials and Methods

### 2.1 Cell culture and maintenance

Cell lines used for  $Ap_4A$  measurements were AA8, EM9 and EM7 [33], H9T3-7-1 [34], NM3 [35], XR-1 and CHO-K1 Chinese hamster (CHO) cells [36], wild type (wt) and *Xrcc1*<sup>-/-</sup> and *Parp1*<sup>-/-</sup> mouse embryo fibroblasts (MEFs) [37], PF20 (wt) and PFL13-*Lig1*<sup>-/-</sup> MEFs [38], Cre4 (wt) and Cre4/2491#6 (*Lig3*<sup>-/-</sup>) MEFs [39], and HeLa, FD105-M20 and FD105-M21 human cells [40]. These were cultured as previously described [35] with minor modifications. Briefly, cells were maintained at 37°C in 5% (v/v) CO<sub>2</sub>/air in Dulbecco's modified Eagle's media (DMEM, Sigma), supplemented with 10% (v/v) foetal bovine serum (Autogene-Bioclear), 1% (v/v) non-essential amino acids, 100  $\mu\text{g mL}^{-1}$  penicillin-streptomycin (Sigma). Cre4/2491#6 (*Lig3*<sup>-/-</sup>) MEFs were also supplemented with 50  $\mu\text{g mL}^{-1}$  uridine [39]. For subculture, cells approaching confluence were washed with 10 mL PBS then removed using 10 mL 0.12% (w/v) trypsin, 0.008% (w/v) EDTA. Trypsinized cells were then added to 10 mL medium and centrifuged at 500 g for 5 min. Supernatants were removed and pelleted cells resuspended in fresh culture medium and reseeded at approximately  $1 \times 10^6$  cells per 75 cm<sup>2</sup> flask.

## 2.2 Growth inhibition assays

Cells were grown in 100 mm dishes until 40-50% confluent, then treated with the stated concentrations of agents for 18 h. They were then trypsinized, centrifuged, resuspended in fresh media and counted. For each MMC concentration,  $5 \times 10^5$  cells were seeded into 75 cm<sup>2</sup> culture flasks containing 15 mL growth medium and grown until the untreated control cultures reached confluence (72-96 h). Cells were then trypsinized and counted using a hemocytometer. Average counts were normalized to the cell count of the untreated culture.

## 2.3 Nucleotide extraction and assay

This was based on our previous method [41]. For each determination, duplicate 100 mm dishes with ~80% confluent cells were used to measure cell numbers and a further three dishes were used for nucleotide extractions and subsequent Ap<sub>4</sub>A assay. Cells were washed with 10 mL PBS then 3 mL ice-cold trichloroacetic acid (TCA) added to each dish. Cells were removed from the dishes with a rubber scraper then transferred to a cold glass tube. The dishes were rinsed with a further 2 mL TCA which was then combined with the extract. The 5 mL combined extract was incubated for 15 min at 4°C then neutralized by adding 5 mL 0.6 M tri-*N*-octylamine in 1, 1, 2-trichlorotrifluoroethane and shaking intermittently for 5 min. Following centrifugation at 500 g for 5 min, 4.4 mL of the upper aqueous layer was removed, and 110 µL 2 M Tris-HCl, pH 8.5, 0.2 M magnesium acetate plus 2 µL (10 U) Antarctic phosphatase (New England Biolabs) added. The mixture was incubated at 18 h at 37°C to degrade ATP completely. To collect and concentrate the resistant nucleotides, 100 µL 50% (v/v) DEAE-Sephacel (Sigma) in 20 mM Tris-HCl, pH 7.6, was added to the extract and the suspension mixed on a rotating platform for 30 min. This was then centrifuged at 12000 g for 1 min, the supernatant removed and the pellet washed with 2 mL water. Bound nucleotides were eluted by incubating the pellet with 0.5 mL 1.0 M triethylammonium bicarbonate (TEAB, pH 8.5) for 5 min with shaking. The suspension was then centrifuged for 1 min at 12000 g and the supernatant collected. The elution step was repeated and the combined supernatants vacuum-dried for 2 h at 70°C. The dried nucleotide extracts were finally dissolved in 120 µL assay buffer (25 mM HEPES-NaOH, pH 7.8, 5 mM magnesium acetate). To remove any residual ATP, 2 µL (10 U) Antarctic phosphatase was added and the tubes incubated at 37°C for 18 h, followed by further incubation at 65°C for 15 min to denature the phosphatase. Duplicate 10 µL aliquots were each mixed with 61 µL assay buffer and 50 µL BacTiter-Glo (Promega) and the background luminescence recorded in a Berthold 9507 luminometer at room temperature. Finally, 225 ng recombinant human NUDT2 Ap<sub>4</sub>A hydrolase [42] was added and the change in luminescence due to released ATP recorded.

## 2.4 Analysis of cell extracts by HPLC

Eight 100 mm dishes of cells were grown to 80% confluence and nucleotides extracted as described above. The vacuum-dried samples were each redissolved in 30 µL 1.0 M TEAB (pH 7.5), combined into a single tube and dried again at 70°C for 2 h. The sample was then redissolved in 21 mM ammonium bicarbonate, pH 9.6, and applied to a MonoQ column (GE Healthcare) equilibrated with 21 mM ammonium bicarbonate, pH 9.6. Nucleotides were eluted with a linear gradient of increasing ionic strength from 21 mM to 0.7 M ammonium bicarbonate, pH 9.6, over a total volume of 20 mL at a flow rate of 0.5 mL min<sup>-1</sup>. Fractions (0.5 mL) were vacuum-dried at 70°C for 2 h, redissolved in 0.5 mL water, dried again, then finally redissolved in 60 µL assay buffer and the Ap<sub>4</sub>A content measured as described above.

## 2.5 Synthesis of ADP-ribosylated Ap<sub>4</sub>A

ADP-ribosylated-Ap<sub>4</sub>A (ADPR-Ap<sub>4</sub>A) was synthesized using poly(ADP-ribose) polymerase 1 (PARP-1) according to a previously published method [43]. The reaction (50  $\mu$ L) contained 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 100  $\mu$ M Ap<sub>4</sub>A, 25  $\mu$ M NAD<sup>+</sup>, 5  $\mu$ g histone H1 (Calbiochem), 10  $\mu$ g PARP active DNA (R&D Systems) and 20  $\mu$ g recombinant PARP-1-HSA enzyme (Trevigen). Reactions were incubated for 18 h at 25°C and then the enzyme was deactivated by incubating for 10 min at 95°C followed by centrifugation for 10 min at 9000 g. The supernatant was removed and stored until further analysis.

## 2.6 Cell-free DNA replication

HeLa and mouse 3T3 cells were cultured in Dulbecco's modified Eagles Media (D-MEM), 10% (v/v) foetal calf serum and penicillin/streptomycin/glutamine (all Gibco). 3T3 cells were synchronized in G<sub>0</sub> by contact inhibition and serum depletion followed by release into fresh media [44]. Late G1-phase and S-phase nuclei and S-phase extracts were prepared as described [44-46]. To assess replication initiation or elongation, 5 $\times$ 10<sup>4</sup> late G1- or S-phase 3T3 nuclei were mixed as appropriate with 10  $\mu$ L of S-phase HeLa extract (supplemented with energy regenerating system, dNTPs and biotinylated dUTP) plus the nucleotide under test at 20  $\mu$ M. Reactions were incubated at 37°C for 60 min, and biotin-labelled nascent DNA visualized by fluorescence microscopy after detection with streptavidin-Alexa Fluor 555 (Life Technologies) and counterstained with Hoechst 33258. The proportion of labelled nuclei was quantified by inspection at 1000 $\times$  magnification, and all nuclei with discrete replication foci or intense uniform labelling were scored positive as previously described [45].

## 2.7 siRNA knockdown

Dharmacon ON TARGET *plus* siRNAs (single or pooled) were designed by and purchased from Thermo Scientific. Scrambled and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNAs were routinely used as controls. The lyophilized RNAs were re-suspended in RNase-free water at 10 pmol/ $\mu$ L and stored at -80°C until use. For siRNA knockdown, cells were grown to approximately 40 % confluence in 100 mm dishes. Prior to transfection, 300 pmol siRNA and 30  $\mu$ L Dharmafect 1 (Thermo) were diluted separately in 500  $\mu$ L serum-free DMEM and incubated at room temperature for 5 min then mixed together and incubated for a further 20 min to allow complex formation. Complexes were then added to cells and incubation carried out for 24-48 h. Following incubation either nucleotides (for Ap<sub>4</sub>A assay, as above) or RNA (for measuring knockdown efficiency) were extracted from parallel cultures. RNA extraction was performed using a Qiagen RNeasy mini kit with QIAshredder and cDNA was synthesized using a Bioline Tetro cDNA synthesis kit, both according to the manufacturer's instructions. The cDNA was then quantitated by PCR using Maxima SYBR Green master mix (Thermo) and a StepOnePlus™ Real Time PCR system (Applied Biosystems). The 2<sup>- $\Delta\Delta$ Ct</sup> method was used to determine relative transcript levels [47]. No significant difference was found in efficiency between single and pooled RNAs.

# 3. Results

## 3.1 Ap<sub>4</sub>A responds to DNA damage

In order to determine whether intracellular Ap<sub>4</sub>A is increased in response to sublethal DNA damage, Chinese hamster AA8 cells, mouse embryo fibroblasts (MEFs) and HeLa cells were treated with low levels of the DNA cross-linking agent, mitomycin C (MMC). MMC was chosen as the effect of cross-linking agents on the level of Ap<sub>4</sub>A has not been studied before and as it is very effective at delaying the overall progress of DNA replication [48]. The growth inhibition curves in Fig. 2a showed no inhibition of the growth of AA8 and MEF cells up to a dose of 100 nM, although HeLa cells were more sensitive. Fig. 2(b-d) shows the dose-dependent increase in Ap<sub>4</sub>A in all three cell lines. Untreated AA8 (Fig. 2b) and HeLa (Fig. 2c) cells had a very similar background level of Ap<sub>4</sub>A of 0.6-0.7 pmol/10<sup>6</sup> cells, consistent

with previous measurements [49], and both showed a 7–8-fold increase after treatment with 100 nM MMC. MEFs (Fig. 2d) consistently had a lower background level of about 0.1 pmol/10<sup>6</sup> cells but showed a similar (9-fold) increase after 100 nM MMC. Treatment of AA8 cells with another DNA cross-linker 1,2,3,4-diepoxybutane (DEB) led to a somewhat lower, 3-fold increase at a dose of 10  $\mu$ M which resulted in 35% growth inhibition (Fig. 3). Given a typical mammalian cell volume of 2000  $\mu$ m<sup>3</sup>, an Ap<sub>4</sub>A content of 1 pmol/10<sup>6</sup> cells equates roughly to an intracellular concentration of 0.5  $\mu$ M if uniformly distributed. These data indicate that a significant increase in intracellular Ap<sub>4</sub>A occurs after sublethal DNA damage and so support the view that this is a biologically significant response.

This conclusion is confirmed by measurements of Ap<sub>4</sub>A in viable mutant cell lines that lack various DNA repair proteins and which contain higher than normal levels of endogenous DNA lesions. XRCC1 is an important scaffolding protein for DNA repair complexes [50, 51]. EM9 and EM7 cells are independent derivatives of AA8 that lack functional XRCC1 [52]. Both these lines have greatly elevated (up to 14-fold) Ap<sub>4</sub>A in the absence of exogenous DNA-damaging agents (Table 1). The normal level of Ap<sub>4</sub>A is restored in EM9 cells expressing full-length human XRCC1 (H9T3-7-1 cells), showing that the increase is specifically due to the loss of XRCC1. MEFs lacking XRCC1 or PARP-1 also show elevated Ap<sub>4</sub>A (4- and 9-fold respectively) as do Chinese hamster cells deficient in the DNA ligase IV accessory protein XRCC4 [53] (XR-1, 2.7-fold) or the Fanconi anemia protein FANCG [35] (NM3, 2.7-fold). Human FD105 cells, derived from an individual with Ataxia with Oculomotor Apraxia type 1 (AOA1) and thus lacking aprataxin [54, 55], show a 5.7-fold increase. Thus, elevated Ap<sub>4</sub>A is not associated with lethal stress but with tolerable levels of DNA damage.

The related nucleotide Ap<sub>3</sub>A has been reported to be the major ligand of the FHIT tumour suppressor protein, which may have a role in the DNA damage response [19, 56]. So, in order to determine whether Ap<sub>3</sub>A responded to DNA damage in a similar manner, it was separately measured in a coupled luminometric assay [57]. No increase in Ap<sub>3</sub>A was found either after DNA damage or, for example, in EM9 cells (2.98  $\pm$  0.32 pmol/10<sup>6</sup> cells, *n*=3) compared to 3.05  $\pm$  0.27 pmol/10<sup>6</sup> cells (*n*=3) in AA8 cells.

### 3.2 Enzymology of Ap<sub>4</sub>A generation *in vivo*

Various enzymes have the ability to synthesize Ap<sub>4</sub>A and several of these, principally the aminoacyl-tRNA synthetases, are thought to contribute to the background level of Ap<sub>4</sub>A found in all cells [14-16]. Less is known about the enzymes responsible for stress-induced increases in Ap<sub>4</sub>A but within the context of DNA replication and repair, DNA ligases are likely candidates and have been proposed to fulfil this role [2]. These enzymes synthesize Ap<sub>4</sub>A by transfer of AMP from the enzyme-adenylate intermediate to an ATP acceptor [15, 58], an activity that is inhibited by DNA-binding [2]. So far, Lig III is the only mammalian ligase shown to synthesize Ap<sub>4</sub>A *in vitro* [2]. We found that the Lig I-specific inhibitor, L82, which prevents DNA-binding but not adenylation activity [59], caused only a slight, 1.3-fold increase in the level of Ap<sub>4</sub>A in AA8 cells treated for 18 h (0.75 pmol/10<sup>6</sup> treated cells *vs.* 0.57 pmol/10<sup>6</sup> untreated cells, *n* = 4) whereas the Lig III inhibitor L67, which acts by a similar mechanism, led to a 5-fold increase in Ap<sub>4</sub>A to 2.72 pmol/10<sup>6</sup> cells, suggesting that Lig III may synthesize Ap<sub>4</sub>A *in vivo* when prevented from associating with DNA even in the absence of DNA damage.

To investigate this further, we examined the levels and MMC-inducibility of Ap<sub>4</sub>A in *Lig I*<sup>-/-</sup> and *Lig 3*<sup>-/-</sup> knockout MEFs on the assumption that knockout of the responsible enzyme would prevent any MMC-induced increase in Ap<sub>4</sub>A. As previously observed, wild type PF20 and Cre4 MEFs had low levels of Ap<sub>4</sub>A (0.12 – 0.15 pmol/10<sup>6</sup> cells). Treatment of PF20 cells with 100 nM MMC increased this level 17-fold to 2.60 pmol/10<sup>6</sup> cells as expected (Table 2). Deletion of Lig I (PFL13) caused a slight, 1.6-fold increase in background Ap<sub>4</sub>A but had no effect on the level reached after treatment with MMC (2.57 pmol/10<sup>6</sup> cells), indicating that Lig I could not have been responsible for this increase. Cre4 MEFs were unusually resistant to MMC, only displaying a 2-fold increase after MMC treatment; however, no significant increase was observed in the *Lig 3*<sup>-/-</sup> knockout derivative Cre4/2491#6 after treatment with MMC, a condition that leads to a very large increase when combined with depletion of other DNA repair proteins (e.g. data in Table 3). The higher background level of Ap<sub>4</sub>A in Cre4/2491#6 cells compared to

wild type Cre4 suggests that the systems responsible for background and MMC-induced levels are not necessarily the same. Moreover, there is evidence that DNA ligases can substitute for one another in ligation reactions [60-62] and the same may be true in relation to Ap<sub>4</sub>A synthesis in ligase knockout cells. Nevertheless, these results implicate Lig III as the most likely, if not sole ligase, contributing to MMC-enhanced Ap<sub>4</sub>A synthesis.

With regard to possible alternative systems, aminoacyl-tRNA synthetases (ARS), some of which are known to exist in nuclei [63], synthesize Ap<sub>4</sub>A *in vitro* by adenylation of ATP by an aminoacyl-AMP, with LysRS being one of the most efficient [64]. LysRS has been implicated in transcription factor activation by synthesizing Ap<sub>4</sub>A, which then disrupts the interactions between MITF and USF2 and the inhibitory Hint1 protein [13, 65]. Due to its unique ability to synthesize Ap<sub>4</sub>A in the absence of glycine by direct adenylyl transfer from one ATP to another, GlyRS has been proposed to be of particular importance in Ap<sub>4</sub>A homeostasis [66] and has been found loosely associated with protein complexes containing DNA polymerase- $\alpha$  [67]. Therefore, the effect of siRNA knockdown of LysRS and GlyRS expression in EM9 cells was studied. Rather than eliminating it, the already high level of Ap<sub>4</sub>A in EM9 cells (4.06 pmol/10<sup>6</sup> cells in this experiment) was actually increased a further 3.7–3.8-fold by knockdown of either ARS (Table 3) possibly as a general response to the suppression of protein synthesis. Thus, these results do not support a role for LysRS or GlyRS in generating the high level of Ap<sub>4</sub>A in EM9 cells.

Another mechanism by which Ap<sub>4</sub>A could be increased after DNA damage is through suppression of the activity of a hydrolytic activity such as the NUDT2 Ap<sub>4</sub>A hydrolase [17]. However, we have found no significant difference in the activity of NUDT2 between AA8 and EM9 cells when cell extracts were subjected to gel filtration chromatography and assayed for NUDT2 activity in the 16-20 kDa region or when AA8 cells were treated with MMC (Fig. 4). Nevertheless, normal NUDT2 expression does appear to limit the extent of Ap<sub>4</sub>A accumulation after DNA damage. Significantly higher levels of Ap<sub>4</sub>A were found in EM9 cells after siRNA knockdown of NUDT2 — a 2.5-fold increase from 4.06 to 9.93 pmol/10<sup>6</sup> cells in EM9 cells alone and a large 16-fold increase to 64 pmol/10<sup>6</sup> cells after MMC treatment (Table 3). Similar results were found with AA8 cells, although the differences were less marked due to the unexplained lower level of knockdown consistently found with AA8 compared to EM9 cells.

Aprataxin, whose major function is to deadenylate abortive DNA ligation intermediates [54], particularly those arising through ribonucleotide excision repair [68], also has a limited ability to degrade Ap<sub>4</sub>A [20]. We have found a 6-fold increase in Ap<sub>4</sub>A in the mutant *APT<sup>X</sup><sup>-/-</sup>* human cell line FD105-M20 compared to FD105-M21 cells, which have been corrected by APTX expression [40] (Table 1), while knockdown of aprataxin expression in EM9 cells led to an 8-fold increase to 31.4 pmol/10<sup>6</sup> cells (Table 3). APTX knockdown also greatly enhanced the MMC-induced increase in AA8 cells from 3.9 to 10.6 pmol/10<sup>6</sup> cells despite the limited reduction in expression. However, it is not clear whether the loss of APTX-mediated Ap<sub>4</sub>A hydrolysis is directly responsible for the increase in Ap<sub>4</sub>A or whether this is due to Lig III-mediated Ap<sub>4</sub>A synthesis in response to an increased level of unrepaired strand breaks in APTX-deficient cells.

Note that none of the findings reported in Table 3 appear to be due to off-target or non-specific effects of the procedure as control data obtained using either scrambled or GAPDH siRNAs were never significantly different from those obtained without RNA addition.

### 3.3 Ap<sub>4</sub>A is ADP-ribosylated *in vivo*

The assay used to measure Ap<sub>4</sub>A relies on the specificity of the NUDT2 Ap<sub>4</sub>A hydrolase, which generates ATP from Ap<sub>4</sub>A [57]. NUDT2 would, however, also generate ATP from Ap<sub>5</sub>A and Ap<sub>6</sub>A, although neither of these nucleotides has even been reported in typical mammalian cells — they appear to be confined to certain excretory granules, such as adrenal chromaffin granules and platelet dense granules [2]. Nevertheless, extracts of AA8 and EM9 cells were subjected to ion-exchange chromatography to investigate further the nucleotide specificity of the response. Surprisingly, in addition to a peak of Ap<sub>4</sub>A (peak 1), a second major (peak 2) and third minor peak (peak 3) that responded to the Ap<sub>4</sub>A assay were

found in EM9 extracts (Fig. 5c), neither of which co-chromatographed with Ap<sub>5</sub>A or Ap<sub>6</sub>A standards (Fig. 5a). The first peak was also seen in AA8 extracts, although relative to Ap<sub>4</sub>A it was very much smaller than that seen in EM9 cells (Fig. 5b); however they both increased significantly along with Ap<sub>4</sub>A after MMC treatment (Fig. 5d). Thus, these additional species appear to respond strongly to DNA damage.

It is known that Ap<sub>4</sub>A can be ADP-ribosylated *in vitro* by PARP1 to produce derivatives with multiple ADP-ribosyl units [43, 69], but such compounds have never been detected *in vivo*. So, in order to determine whether peaks 2 and 3 might represent ADP-ribosylated derivatives, mono- and di-ADP-ribosylated Ap<sub>4</sub>A (ADPR-Ap<sub>4</sub>A) were synthesized according to published procedures [43]. These synthetic compounds were authenticated by mass spectrometry ( $m/z = 1371.64$  and  $1918.75$  respectively) and found to co-chromatograph with peaks 2 and 3 respectively (Fig. 6a). Furthermore, when EM9 cells were treated *in vivo* with the PARP inhibitor KU-0058948 [70], the subsequent dinucleotide extract had a greatly reduced peak 2 and no peak 3 (Fig. 6b, compare to Fig. 5c), suggesting that a KU0058948-sensitive PARP was involved in their generation. Furthermore, when an EM9 extract was treated *in vitro* with poly(ADP-ribose) glycohydrolase (PARG) and then chromatographed, peaks 2 and 3 were abolished and the Ap<sub>4</sub>A peak increased, indicating a release of assayable Ap<sub>4</sub>A from ADP-ribosylated species by removal of the ADP-ribose (Fig. 6c, compare to Fig. 5c). Finally, treatment of EM9 cells *in vivo* with the PARG inhibitor gallotannin [71] increased peaks 2 and 3, especially peak 3, at the expense of Ap<sub>4</sub>A (Fig. 6d). Taken together, these results clearly show that survivable DNA damage leads to an increase in the level of Ap<sub>4</sub>A and the appearance of its mono- and di-ADP-ribosylated derivatives (Fig. 1b). So far we have not detected any longer poly(ADP-ribosyl)ated species *in vivo*.

KU-0058948 is a specific inhibitor of PARP-1 and PARP-2, being one to three orders of magnitude more effective against these enzymes compared to other ADP-ribosyltransferases tested [70]. Therefore, the data in Fig. 6b, in which KU-0058948 led to a dramatic drop in the (mono + di)ADPR-Ap<sub>4</sub>A:Ap<sub>4</sub>A ratio in EM9 cells from 1.45 to 0.045 (equivalent to a 97% reduction in the ADP-ribosylated species), would suggest that one of these PARPs is responsible for the ADP-ribosylation of Ap<sub>4</sub>A. To investigate this further, the ADPR-Ap<sub>4</sub>A:Ap<sub>4</sub>A ratio was measured in *Parp1*<sup>-/-</sup> knockout MEFs after treatment with 100 nM MMC. This ratio was 0.19 in the knockouts compared to 0.33 in the wild type cells, indicating a 40% decrease in ADPR-Ap<sub>4</sub>A in the absence of PARP-1 (Table 4). The ratio was further reduced to 0.12 when PARP2 expression was knocked down by siRNA in the *Parp1*<sup>-/-</sup> knockout MEFs. The ADPR-Ap<sub>4</sub>A:Ap<sub>4</sub>A ratio was used to quantify the results because of the increase in the absolute levels of both nucleotides in PARP-deficient cells due to retarded repair of endogenous DNA damage. Thus it appears that both PARP1 and PARP2 are able to ADP-ribosylate Ap<sub>4</sub>A although their relative contributions in wild type cells is harder to assess. As with DNA ligases, there is evidence that PARP1 and PARP2 may exhibit some functional redundancy in DNA repair processes [72].

### 3.4 Ap<sub>4</sub>A inhibits the initiation of DNA replication

It has previously been suggested, without supporting evidence, that elevated Ap<sub>4</sub>A might act as a stress-induced alarmone to either inhibit DNA replication to allow repair to occur or to promote replication fork restart [2-4, 21]. Therefore, in order to assess whether Ap<sub>4</sub>A could inhibit DNA replication, we utilized an *in vitro* DNA replication assay that has previously been used to recapitulate either the elongation phase or the initiation phase of DNA replication using defined synchronized nuclei and cytosolic extracts [46, 73-75]. Incubation of a G1 extract and late G1 nuclei [44] reveals the population of cells currently in S-phase due to asynchrony (Fig. 7a, 12 %); initiation competent nuclei are identified by addition of a cytosolic S-phase extract that increases the number of S-phase nuclei to 45% of the population (Fig. 7a). Using this approach to test whether Ap<sub>4</sub>A could affect initiation, elongation, or both, G1 nuclei and S-phase extracts were initially incubated with 20  $\mu$ M Ap<sub>4</sub>A. This revealed a marked reduction in the number of nuclei undergoing DNA replication (22%, Fig. 7a) suggesting that Ap<sub>4</sub>A inhibits some aspect of this process. This result could conceptually be caused by a failure of cells to initiate replication *de novo* or via inhibition of the elongation phase.



Therefore, to assess whether Ap<sub>4</sub>A affects the elongation phase, an S-phase population of cells was produced by a double thymidine block [46, 73-75]. Under these conditions, 100% of nuclei were in S-phase in control reactions and addition of up to 1 mM Ap<sub>4</sub>A did not reduce the number of replicating nuclei, suggesting that Ap<sub>4</sub>A does not inhibit DNA replication elongation (Fig. 7b). This contrasts with reactions that recapitulate the latter stages of G1 phase and monitor initiation of DNA replication using replication-licensed late G1-phase nuclei and S-phase cytosolic extracts [44, 46, 73]. Under these conditions, Ap<sub>4</sub>A caused a marked, dose-dependent reduction in DNA replication maximizing at 70-80% inhibition at 20  $\mu$ M Ap<sub>4</sub>A that was maintained up to 1 mM Ap<sub>4</sub>A (Fig. 7c). To ensure that this effect was specific to Ap<sub>4</sub>A, reactions were also performed with Ap<sub>3</sub>A, Ap<sub>5</sub>A, Gp<sub>4</sub>G and ADPR-Ap<sub>4</sub>A, which revealed no significant effect of these Ap<sub>4</sub>A analogs at 20  $\mu$ M (Fig. 7d). Even at 0.8  $\mu$ M Ap<sub>4</sub>A (approx. 1.6 pmol/10<sup>6</sup> cells) inhibition was significant at 40%. The inhibitory concentration of Ap<sub>4</sub>A seen here is consistent with that found after induction with sub-lethal doses of DNA crosslinking agents, suggesting that damage-induced Ap<sub>4</sub>A could retard the initiation of DNA replication at physiologically relevant levels. As cells, e.g. EM9, are perfectly able grow in the presence of elevated Ap<sub>4</sub>A this inhibition must be readily reversed *in vivo*; the *in vitro* system used here has revealed its existence for further analysis.

#### 4. Discussion

Previous DNA damaging agents shown to increase intracellular Ap<sub>4</sub>A in mammalian cells have included *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), bleomycin, and either ultraviolet (UV) light or 4-nitroquinoline-*N*-oxide (4NQO) in the presence of arabinosylcytosine (araC) [5, 6, 22]. Since Ap<sub>4</sub>A did not increase in incision-defective xeroderma pigmentosum complementation group A cells treated with UV or 4NQO plus araC, it was suggested that Ap<sub>4</sub>A accumulated in response to direct or indirect DNA strand breaks [22]. The response to DNA cross-linking agents has not been studied before. Here we have shown that non-cytotoxic levels of mitomycin C and diepoxybutane are highly effective at increasing Ap<sub>4</sub>A. As the repair of these lesions will also lead to indirect strand breaks, these data along with the enhanced levels found in cells lacking XRCC1, PARP1 and FANCG, in which endogenous strand breaks are likely to have a prolonged half-life, would support the role of strand breaks as at least one causative factor. However, interstrand crosslinks may themselves be a more potent stimulus, which would explain why Ap<sub>4</sub>A increases significantly at low doses of MMC compared to the more toxic doses of monofunctional alkylating agents previously used. The higher efficacy of MMC compared to DEB may be due to the ability of MMC crosslinks to block replication with minimal perturbation of the DNA structure and their consequent poor removal by nucleotide excision repair mechanisms outside S-phase [76]. Thus, increased Ap<sub>4</sub>A is not necessarily associated with excessive stress and cell death, as has previously been suggested, and so this response is likely to be biologically relevant.

Although ARSs are generally held to be major contributors to the intracellular Ap<sub>4</sub>A pool, GlyRS and LysRS at least do not seem to be responsible for the DNA damage-induced increases observed here and we have no *a priori* reason to implicate other members of this ligase family. Knockdown of GlyRS and LysRS expression led in fact to an unexpected increase in Ap<sub>4</sub>A in EM9 cells. LysRS has been shown to be responsible for the stimulation of Ap<sub>4</sub>A synthesis by immune complexes in mast cells and its knockdown in that case completely eliminated Ap<sub>4</sub>A induction [13]. Thus, the facts that LysRS-mediated Ap<sub>4</sub>A synthesis can be abolished by siRNA knockdown but that knockdown does not eliminate DNA damage-mediated Ap<sub>4</sub>A synthesis would support our contention that ARSs are not responsible for the DNA damage-mediated response. ARS knockdown is known to cause translational suppression [77] and this may have served as the stimulus for increased Ap<sub>4</sub>A synthesis by other synthetases independently of DNA damage. Although data are lacking in eukaryotes, Ap<sub>4</sub>A has been proposed as a signal for translational incapacity in *E. coli* [78] and the increases in ARS knockdown cells are consistent with this additional role for Ap<sub>4</sub>A. One other ARS of special interest in nuclei is TrpRS as it has been found associated with protein complexes containing DNA polymerase- $\alpha$  and the unidentified Ap<sub>4</sub>A-binding

protein [67] and with DNA-dependent protein kinase and PARP-1 [79]. However, TrpRS is unusual in being only able to synthesize Ap<sub>3</sub>A, and not Ap<sub>4</sub>A [80, 81], and so could not be responsible for the observed increases. Instead, our results would favor a role for DNA ligase III as the main enzyme responsible for the synthesis of DNA damage-induced Ap<sub>4</sub>A.

Although our data support stimulation of Ap<sub>4</sub>A synthesis as a major cause of increased Ap<sub>4</sub>A, an additional role for decreased degradation cannot be excluded. The association of APTX with XRCC1, PARP1 and XRCC4 in DNA repair complexes [82, 83] makes it an ideal candidate for such a role and we have found that cells from individuals with AOA1, which lack functional APTX, do have significantly increased Ap<sub>4</sub>A and that APTX knockdown increases Ap<sub>4</sub>A in hamster cells. However, further analysis using specific APTX mutants will be required to determine whether this is caused by loss of the Ap<sub>4</sub>A hydrolase activity *per se* rather than a secondary effect due to persistent DNA damage in APTX<sup>-</sup> cells. Alterations in the activity of NUDT2, the major intracellular Ap<sub>4</sub>A hydrolase, do not appear to be responsible for DNA damage-induced accumulation of Ap<sub>4</sub>A, although the enzyme does limit the extent of the increase and may prevent harmful levels from being reached. At high levels, Ap<sub>4</sub>A can inhibit the activity of several protein kinases, something that could prove cytotoxic [2]. Interestingly, overexpression of NUDT2 has been correlated with a slight increase in cellular proliferation rate while siRNA-mediated reduction in NUDT2 expression has been associated with slower proliferation [84]. Although the level of Ap<sub>4</sub>A was not measured in that study, these results are consistent with the inhibitory effect of Ap<sub>4</sub>A on DNA replication initiation. Another protein that can hydrolyze Ap<sub>4</sub>A is the Fhit tumor suppressor protein, although it prefers Ap<sub>3</sub>A as a substrate [19]; however, Fhit appears to have no role in controlling the level of Ap<sub>4</sub>A *in vivo* as the level of Ap<sub>4</sub>A is known to be unchanged in Fhit-deficient cells [41].

The hypothesized role for Ap<sub>4</sub>A as an inhibitor of DNA replication is directly supported for the first time by our finding that it inhibits replication in a well-characterized cell-free system comprising G1 nuclei and essential cytoplasmic components [44-46]. In contrast, elongation at previously initiated replication forks in S-phase nuclei appears to be unaffected. Together, these results are consistent with a specific effect on initiation. However, without further experimentation we cannot say at what point initiation is affected and whether this depends, for example, on the presence of endogenous DNA lesions; nor can we exclude an effect on elongation rates or other aspects of replication such as origin usage and inter-origin distance. Nevertheless, the concentrations at which inhibition occurs are well within the range achieved *in vivo*. Inhibition of origin initiation by Ap<sub>4</sub>A is consistent with the observations that (i) fewer new origins are initiated after MMS treatment of XRCC1-defective EM9 cells compared to cells complemented with XRCC1 [85] and (ii) MMC primarily reduces the abundance of replication forks but not rates of fork progression [48]. Whether the unidentified Ap<sub>4</sub>A-binding protein associated with DNA polymerase- $\alpha$  is involved in this inhibition remains to be determined. However, it is interesting to note that transformation of the data in Fig.7b to fit a single rectangular hyperbola gave a good fit ( $R^2 = 0.90$ ) to a single site model with a  $K_i$  of  $0.28 \pm 0.09 \mu\text{M}$ , a figure that agrees well with a previously measured  $K_d$  of  $0.15 \mu\text{M}$  for the HeLa cell Ap<sub>4</sub>A binding protein [23].

The combined PARP inhibitor and knockout data suggest that either or both of PARP1 and PARP2 are responsible for the ADP-ribosylation of Ap<sub>4</sub>A *in vivo*. Of the many known ADP-ribosyltransferases only PARP1, PARP2 and PARP5 (tankyrase) isoforms are known to synthesize ADP-ribose oligomers [86], so the presence of di(ADPR)-Ap<sub>4</sub>A in cell extracts would suggest that none of the many mono-ADP-ribosyltransferases, including PARP3, is responsible. Furthermore, tankyrase is insensitive to KU-0058948 and so can be discounted [70]. However, the function of the mono- and di(ADPR)-Ap<sub>4</sub>A species is still not clear. One possibility is that they serve to inactivate the inhibitory function of Ap<sub>4</sub>A by preventing it from binding to its target(s) and so permit the rapid resumption of DNA replication initiation following lesion repair. One previous study showed that the PARP inhibitor 3-aminobenzamide enhanced the 2-fold increase in Ap<sub>4</sub>A in HTC hepatoma cells caused by  $200 \mu\text{M}$  MNNG by 100% and prevented its subsequent recovery to the basal level over a period of 5 hours [6]. This is consistent with an inactivating function for the ADP-ribosylation. Certainly, inhibition of initiation by Ap<sub>4</sub>A *in vivo* must be transient, as cells possessing high levels of Ap<sub>4</sub>A do proceed successfully into and through S-phase.

Interestingly, synthetic poly(ADP-ribosylated)Ap<sub>4</sub>A with an average chain length of 7.5 ADPR units has been shown to inhibit T-antigen-dependent DNA synthesis from an SV40 origin *in vitro* [87]; however, we have not detected species longer than di(ADPR)-Ap<sub>4</sub>A *in vivo*, so the biological significance of the longer inhibitory polymers remains unresolved.

In conclusion, our results support Varshavsky's original hypothesis that stalled replication complexes generate a low-molecular-weight alarmone signal, Ap<sub>4</sub>A, that aids survival after DNA damage [3, 4]. We propose that DNA ligase III synthesizes Ap<sub>4</sub>A when prevented from accessing ligation sites in the DNA through changes in protein-protein interactions involving XRCC1, PARP1, APTX and other components. Changes in APTX hydrolytic activity may further enhance the increase in Ap<sub>4</sub>A. The resulting Ap<sub>4</sub>A then binds to one or more target proteins and temporarily delays the initiation of new replicons. This would help prevent further replication forks from encountering DNA lesions. One possible advantage of damage-generated Ap<sub>4</sub>A is that it could act as a diffusible signal and so exert its protective effect at secondary sites close to the stalled replication apparatus. ADP-ribosylation of the Ap<sub>4</sub>A may then serve to rapidly terminate its inhibitory function. Although a number of questions remain to be answered, we hope that these findings will serve as a platform for renewed interest in Ap<sub>4</sub>A as an important inducible ligand in the DNA damage response.

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## Figure legends

**Fig. 1.** Structures of (a) Ap<sub>4</sub>A and (b) ADP-ribosylated Ap<sub>4</sub>A derivatives; n=1, mono-ADPR-Ap<sub>4</sub>A; n=2, di-ADPR-Ap<sub>4</sub>A. The position of the linkage between Ap<sub>4</sub>A and ADP-ribose has not been unequivocally established but is assumed based on the linkage between ADP-ribose units in poly(ADP-ribose) [77].

**Fig. 2.** Effect of mitomycin C (MMC) on the growth of AA8, MEF and HeLa cells. Cells were treated for 18 h with the indicated dose of MMC after which they were (a) counted and plated into 75 cm<sup>2</sup> flasks to measure growth or (b-d) subjected to nucleotide extraction and Ap<sub>4</sub>A measurement as described in Materials and Methods.

**Fig. 3.** Effect of 1,2,3,4-diepoxybutane (DEB) on AA8 cells. Cells were treated for 18 h with the indicated dose of DEB after which they were (a) counted and plated into 75 cm<sup>2</sup> flasks to measure growth or (b) subjected to nucleotide extraction and Ap<sub>4</sub>A measurement as described in Materials and Methods.

**Fig. 4.** Activity of NUDT2 Ap<sub>4</sub>A hydrolase in (a) AA8 and EM9 cells and (b) AA8 cells treated with 100 nM and 1  $\mu$ M mitomycin C. Chromatography is necessary to remove contaminating ATPases and phosphodiesterases that interfere with the assay. Extracts of AA8 and EM9 cells were prepared and 2 mg soluble protein in 0.7 ml 50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 1% (v/v) Igepal CA-630 applied and run at 1 mL min<sup>-1</sup> on a 16  $\times$  60 HiLoad Superdex 75 column in 50 mM Tris-HCl (pH 7.6), 200 mM NaCl. Fractions (1 mL) were collected and 20  $\mu$ L assayed for NUDT2 Ap<sub>4</sub>A hydrolase activity. The column was calibrated with molecular weight standards: (1) dextran blue (void volume), (2) BSA (67 kDa), (3) ovalbumin (43 kDa), (4) chymotrypsinogen A (25 kDa) and (5) ribonuclease A (13.7 kDa).

**Fig. 5.** High performance ion-exchange chromatography of cellular dinucleotide extracts. Standards (a) and extracts of (b) AA8, (c) EM9 and (d) AA8 cells treated for 18 h with 100 nM MMC were subjected to hplc analysis and fractions assayed for Ap<sub>4</sub>A as described in Materials and Methods.

**Fig. 6.** High performance ion-exchange chromatography of dinucleotide preparations. Samples were (a) 50  $\mu$ L of ADPR-Ap<sub>4</sub>A synthetic reaction prepared as described in Materials and Methods; (b) extract of EM9 cells grown in the presence of 0.1  $\mu$ M KU0058948 for 18 h; (c) extract of EM9 cells incubated for 18 h with 10  $\mu$ g human PARG (Enzo Life Sciences); (d) extract of EM9 cells grown in the presence of 100  $\mu$ M gallotanin (Enzo) for 18 h.

**Fig. 7.** Effect of Ap<sub>4</sub>A and related nucleotides on DNA replication in a cell-free DNA replication system. (a) Reconstitution of the initiation phase of DNA replication. G1 extract and G1 nuclei reveal the proportion of cells in S-phase in this population. Replication licensed nuclei that are initiation competent can be stimulated to enter S-phase by addition of S-phase cytosolic extracts (middle bar) and the effect of 20  $\mu$ M Ap<sub>4</sub>A on DNA replication (right bar) shows a reduction in the number of nuclei incorporating biotinylated-dUTP (b-dUTP). Further statistical analysis of the DNA replication activity after dinucleotide treatment was performed by one-way analysis of variance (ANOVA) using Tukey's test post-hoc at 99.9% significance levels using IBM SPSS statistics 21 (\*\*\* =  $P < 0.001$ ). (b) Increasing concentrations of Ap<sub>4</sub>A (0–1 mM) were incubated for 60 min with S-phase extract, S phase nuclei, b-dUTP and % nuclei incorporating label determined. Data are means  $\pm$  S.D.,  $n = 3$ . (c) Increasing concentrations of Ap<sub>4</sub>A were incubated with S-phase extract, G1 nuclei, b-dUTP and the % nuclei incorporating label due to initiation of replication determined as previously described [73, 74, 76, 77]. Data are means  $\pm$  S.D.,  $n = 3$ . Inset, data as described in (c) and presented in an unscaled format. (d) Various dinucleotides (20  $\mu$ M) were incubated for 60 min with S-phase extract, G1-phase nuclei, b-dUTP as in (c) and the % nuclei incorporating label determined. Data are means  $\pm$  S.D.,  $n = 3$ . Further statistical analysis of the DNA replication activity after dinucleotide treatment was performed by one-way analysis

of variance (ANOVA) using Tukey's test post-hoc at 99.9% significance levels using IBM SPSS statistics 21 where \*\*\* =  $P < 0.001$ .

## Tables

**Table 1.** Intracellular level of Ap<sub>4</sub>A in various cell lines.<sup>a</sup>

Cell line	Ap <sub>4</sub> A (pmol/10 <sup>6</sup> cells)	<i>n</i>
AA8	0.63 ± 0.07	6
EM9 ( <i>Xrcc1</i> <sup>-/-</sup> )	4.16 ± 0.30	6
EM7 ( <i>Xrcc1</i> <sup>-/-</sup> )	8.77 ± 0.84	3
H9T3-7-1 (XRCC1-corrected EM9)	0.78 ± 0.06	4
MEF wt	0.06 ± 0.01	6
MEF <i>Xrcc1</i> <sup>-/-</sup>	0.24 ± 0.09	3
MEF <i>Parp1</i> <sup>-/-</sup>	0.56 ± 0.06	3
CHO-K1 (control for XR-1)	0.62 ± 0.08	3
XR-1 ( <i>Xrcc4</i> <sup>-/-</sup> )	1.73 ± 0.20	3
NM3 ( <i>FANCG</i> <sup>-/-</sup> )	2.98 ± 0.13	3
NM3 ( <i>FANCG</i> corrected)	1.10 ± 0.11	3
FD105 M20 ( <i>APTX</i> <sup>-/-</sup> )	1.26 ± 0.29	3
FD105 M21 ( <i>APTX</i> corrected)	0.22 ± 0.03	3

<sup>a</sup> Cells were grown, extracted and Ap<sub>4</sub>A measured as described in Materials and Methods. Results are means ± S.D.

**Table 2.** Intracellular level of Ap<sub>4</sub>A in DNA ligase-deficient MEFs with and without treatment with 100 nM MMC.<sup>a</sup>

Cell line	MMC	Ap <sub>4</sub> A (pmol/10 <sup>6</sup> cells)
PF20 (wt)	-	0.15 ± 0.04
PF20 (wt)	+	2.60 ± 0.44
PFL13 ( <i>LigI</i> <sup>-/-</sup> )	-	0.25 ± 0.06
PFL13 ( <i>LigI</i> <sup>-/-</sup> )	+	2.57 ± 0.63
Cre4 (wt)	-	0.12 ± 0.02
Cre4 (wt)	+	0.27 ± 0.13
Cre4/2491#6 ( <i>Lig3</i> <sup>-/-</sup> )	-	0.54 ± 0.27
Cre4/2491#6 ( <i>Lig3</i> <sup>-/-</sup> )	+	0.58 ± 0.31

<sup>a</sup> Cells were grown, extracted and Ap<sub>4</sub>A (including ADPR-Ap<sub>4</sub>A) measured as described in Materials and Methods. Results are mean ± S.D. (*n* = 3).

**Table 3.** Effect of siRNA knockdown of the expression of potential Ap<sub>4</sub>A-metabolizing enzymes on the level of intracellular Ap<sub>4</sub>A.<sup>a</sup>

Cell line	RNA target	Ap <sub>4</sub> A (pmol/10 <sup>6</sup> cells)
EM9	-	4.06 ± 0.66
EM9	Scrambled	4.15 ± 0.25
EM9	GAPDH	3.67 ± 0.11
EM9	LysRS	14.81 ± 3.36
EM9	GlyRS	15.42 ± 2.31
EM9	NUDT2	9.93 ± 1.65
EM9	APTX	31.39 ± 3.04
AA8	-	0.63 ± 0.07
AA8	Scrambled	0.56 ± 0.11
AA8	GAPDH	0.90 ± 0.26
AA8	NUDT2	0.66 ± 0.31
AA8	APTX	1.18 ± 0.03
EM9 + MMC	-	17.08 ± 0.16
EM9 + MMC	GAPDH	14.60 ± 1.04
EM9 + MMC	NUDT2	64.19 ± 2.54
EM9 + MMC	APTX	43.62 ± 2.92
AA8 + MMC	-	3.92 ± 0.12
AA8 + MMC	GAPDH	3.49 ± 0.18
AA8 + MMC	NUDT2	4.67 ± 0.14
AA8 + MMC	APTX	10.56 ± 1.88

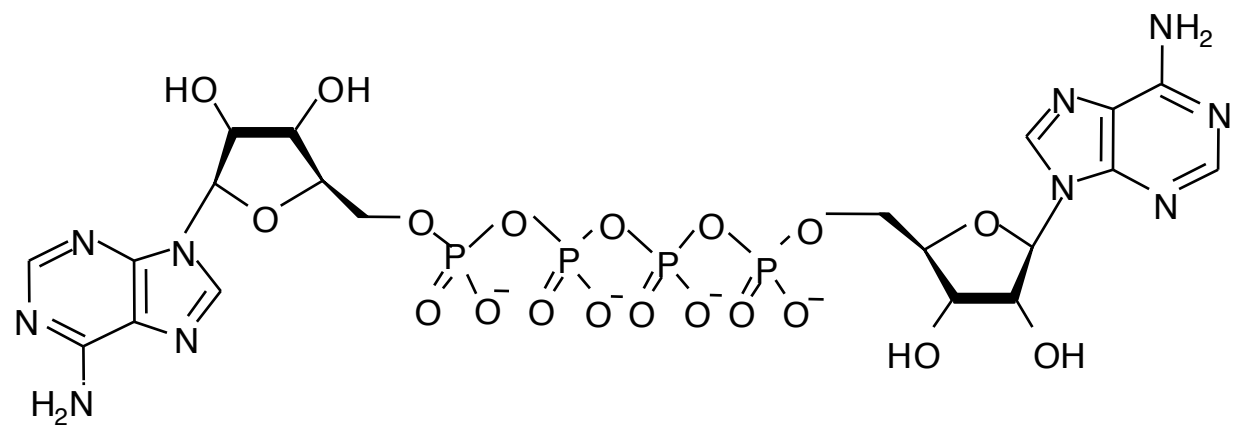
<sup>a</sup>Knockdown was carried out and quantitated by RT-qPCR as described in Materials and Methods and was typically between 70 and 80% for EM9 cells and between 20 and 30% for AA8 cells. The levels of Ap<sub>4</sub>A include ADPR-Ap<sub>4</sub>A. When added, MMC was at 100 nM. Results are mean ± S.D. (*n* = 3).

**Table 4.** Roles of PARP1 and PARP2 in ADP-ribosylation of Ap<sub>4</sub>A in mouse embryo fibroblasts treated with 100 nM MMC.<sup>a</sup>

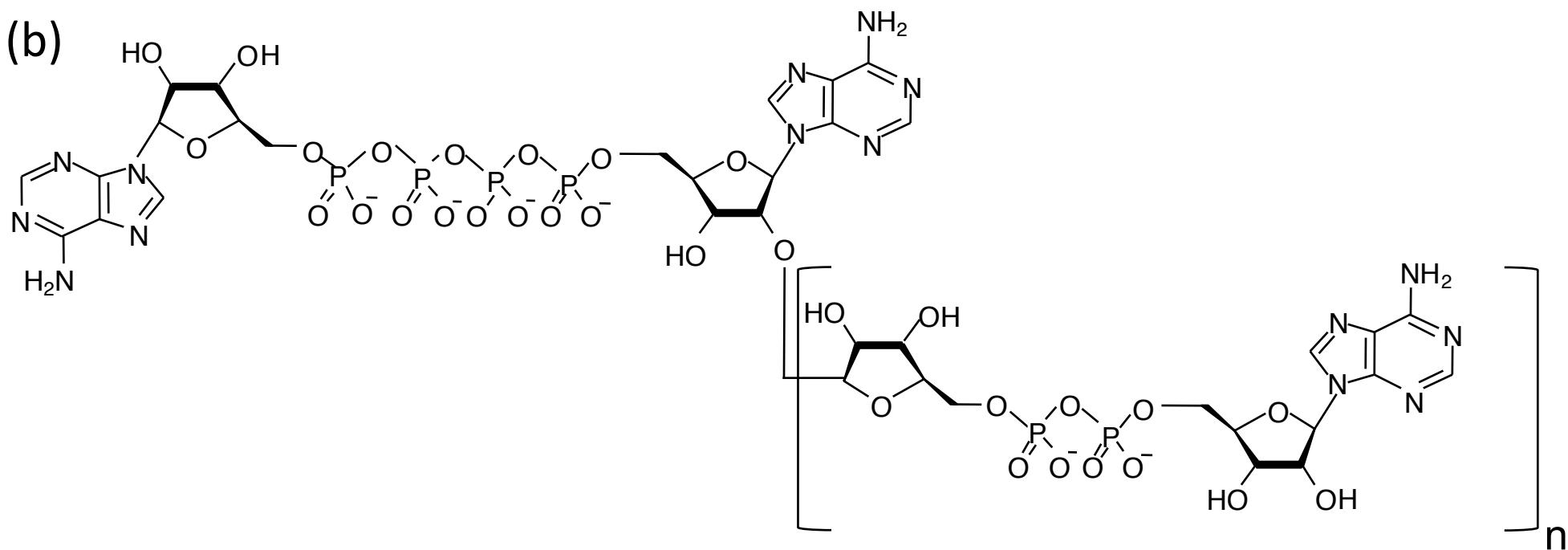
Cell line	Ap <sub>4</sub> A (pmol)	ADPR-Ap <sub>4</sub> A (pmol)	ADPR-Ap <sub>4</sub> A/Ap <sub>4</sub> A
<i>Parp1</i> <sup>+/+</sup> (wt) MEFs	0.261	0.086	0.33
<i>Parp1</i> <sup>-/-</sup> MEFs	0.736	0.140	0.19
<i>Parp1</i> <sup>-/-</sup> MEFs + PARP2 KD	3.63	0.44	0.12

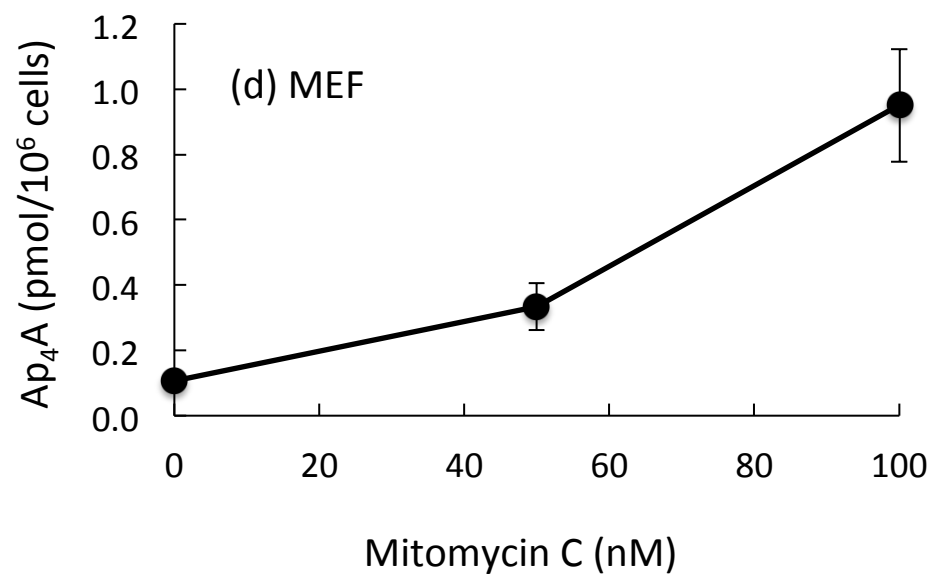
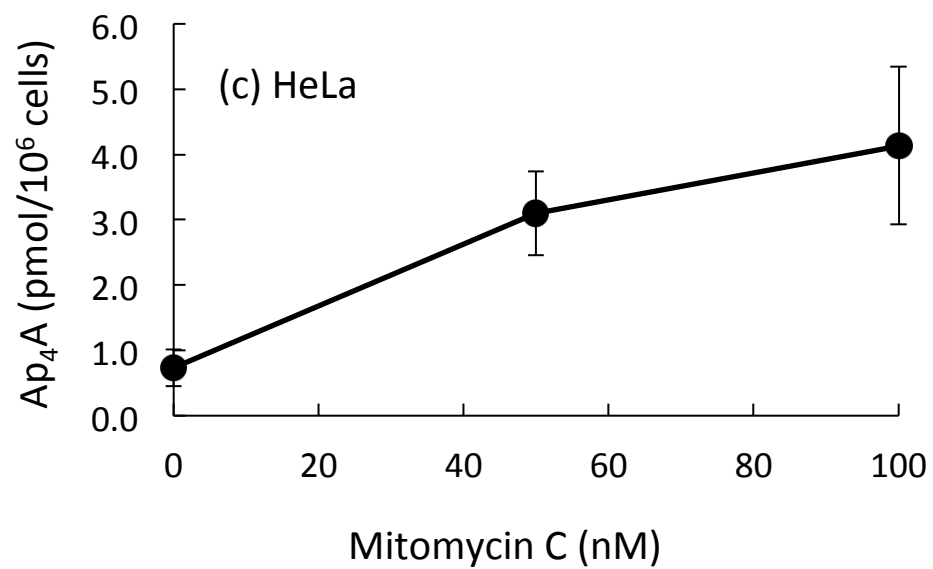
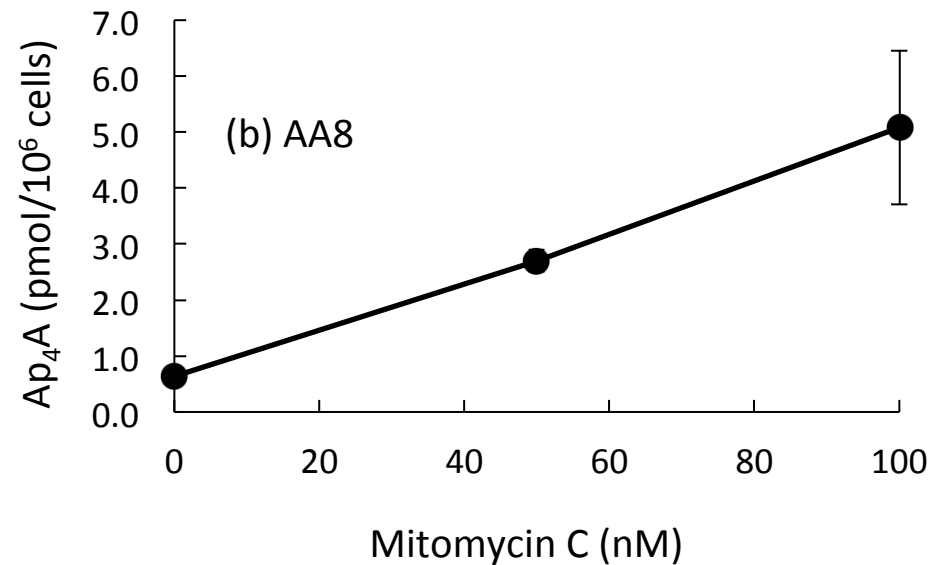
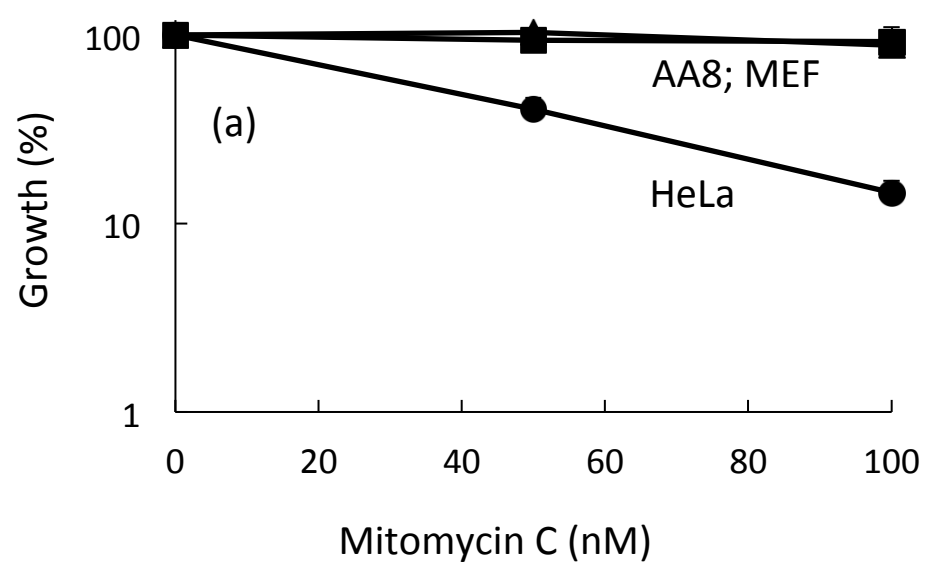
<sup>a</sup>The degree of PARP2 knockdown measured by RT-qPCR was 74%. Cell extracts were subjected to hplc and assay as described in Materials and Methods and the levels of Ap<sub>4</sub>A and ADPR-Ap<sub>4</sub>A summed in the relevant fractions. Results are the means of two independent determinations.

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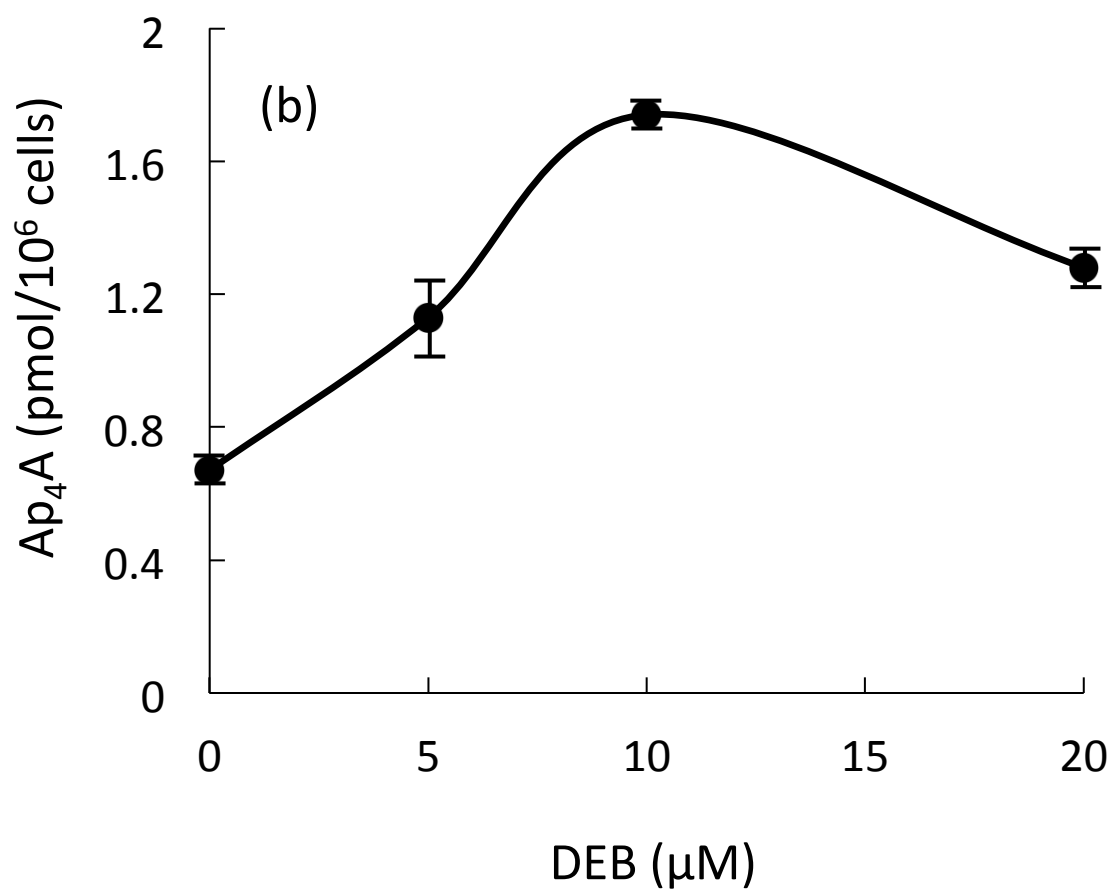
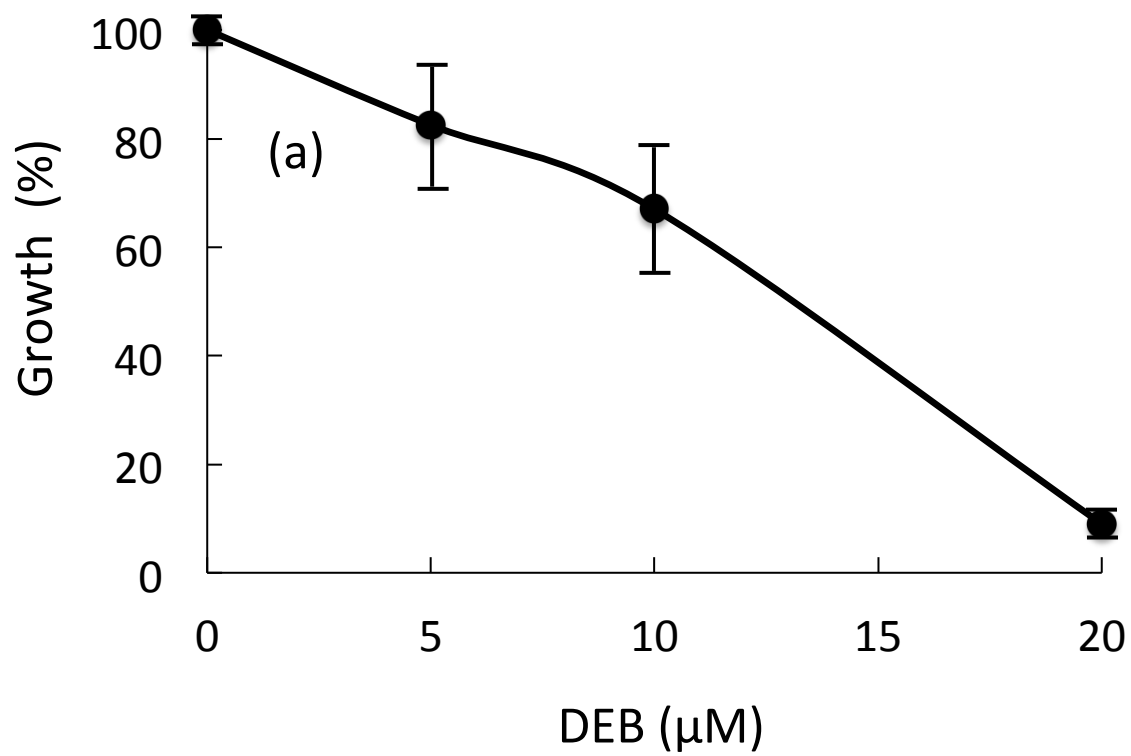


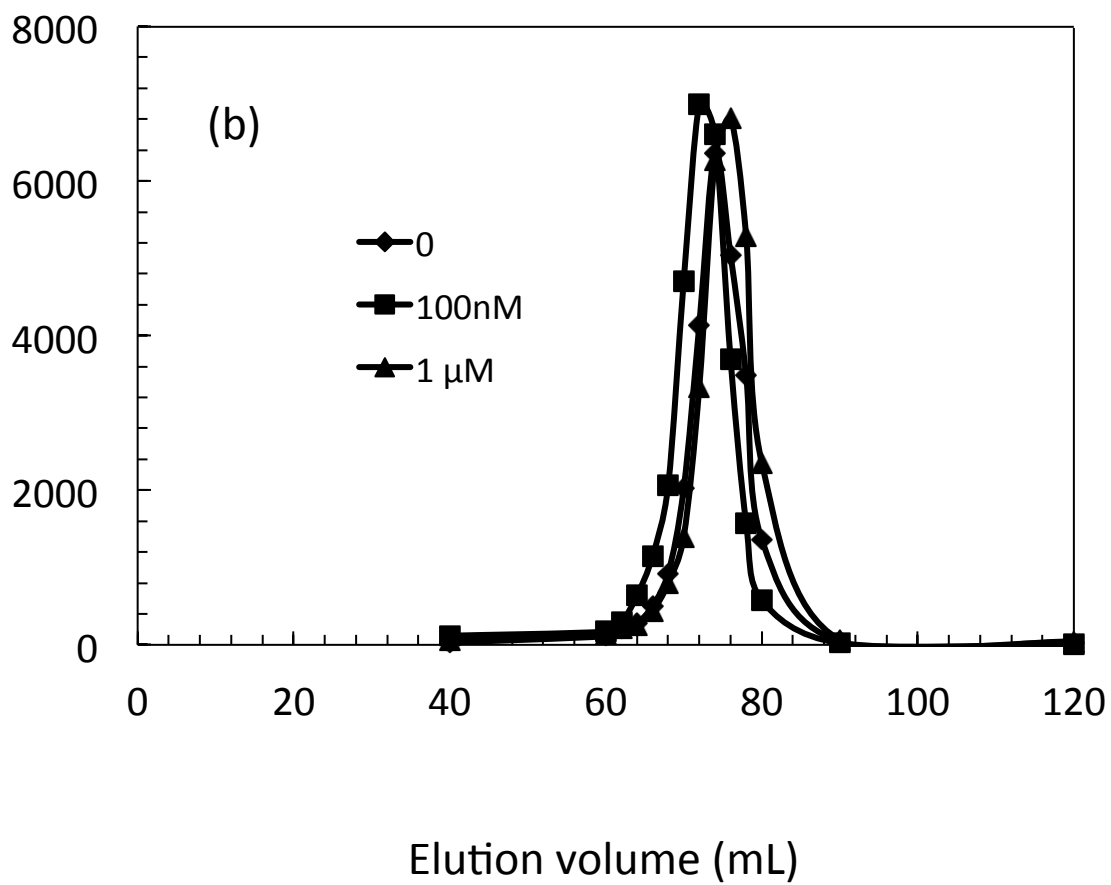
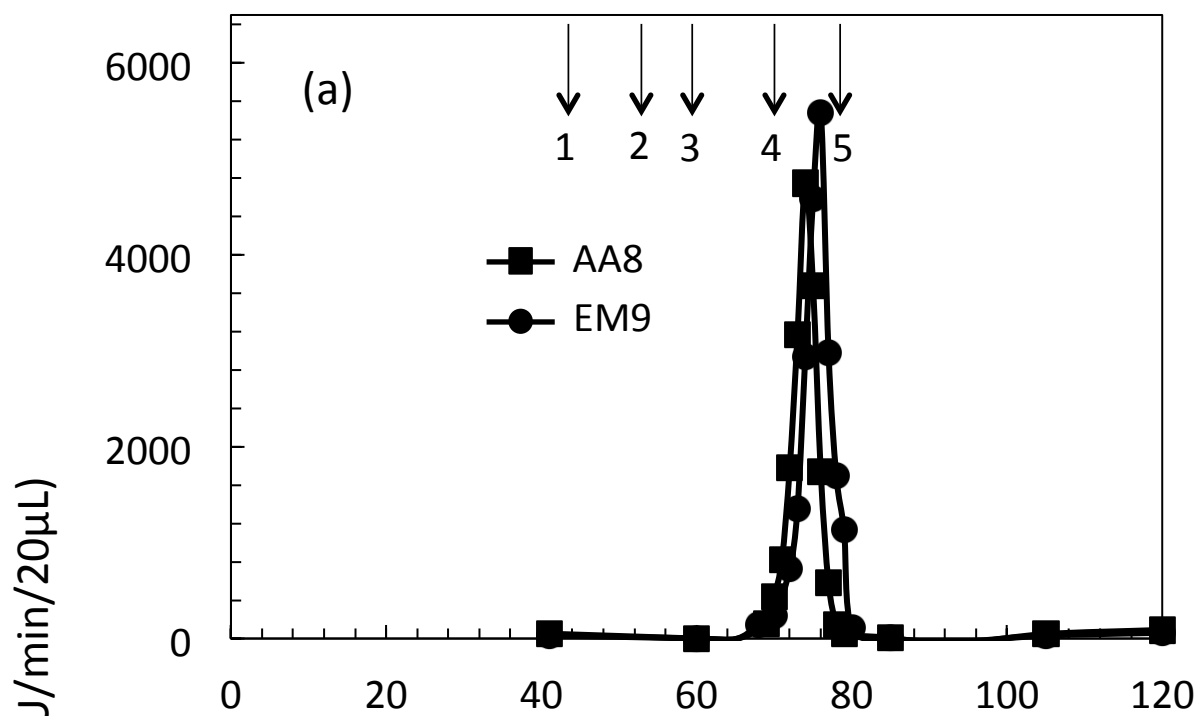
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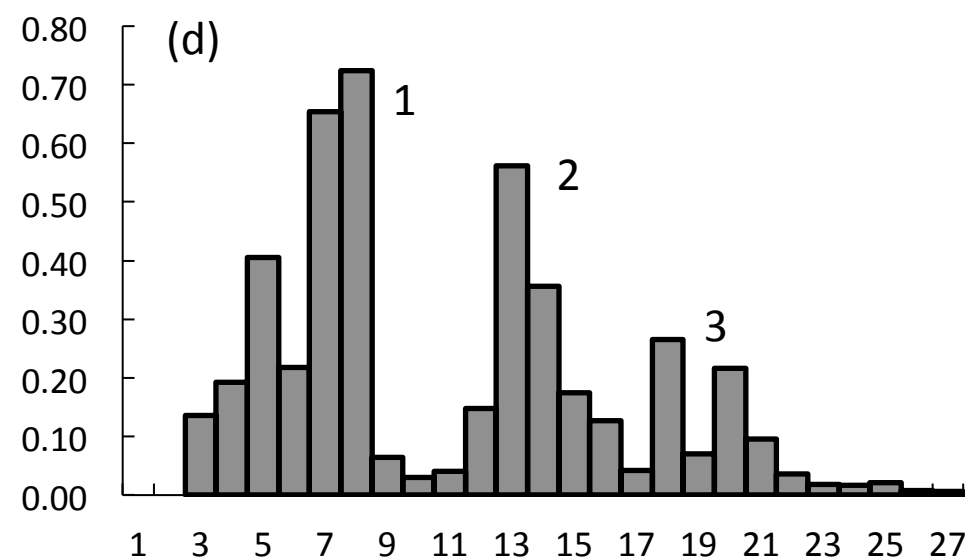
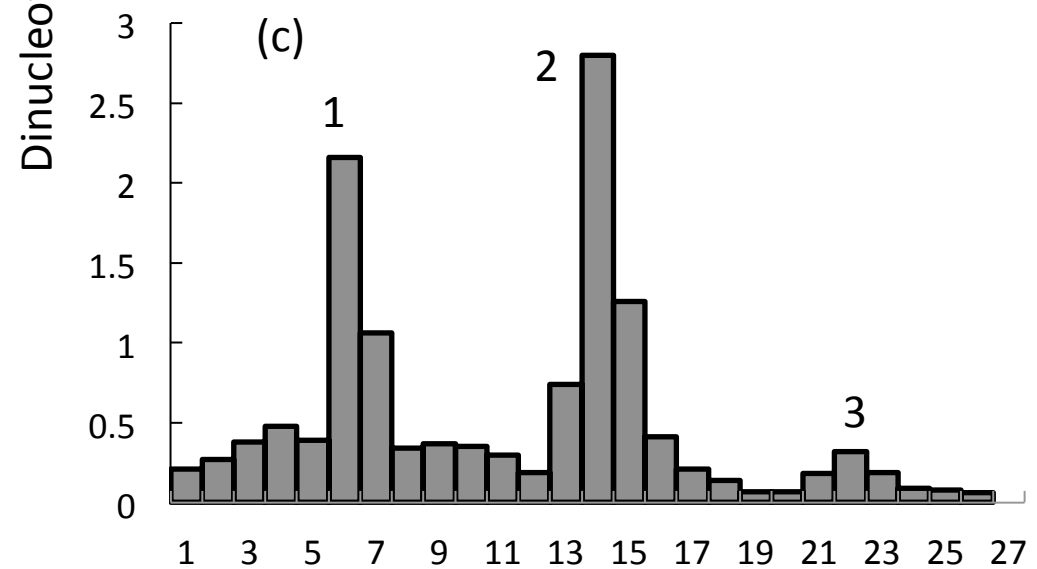
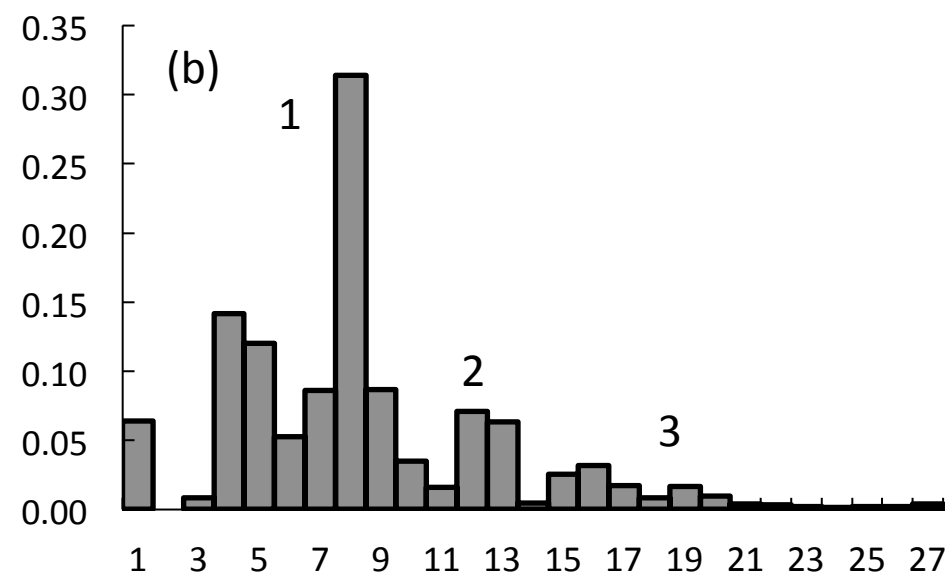
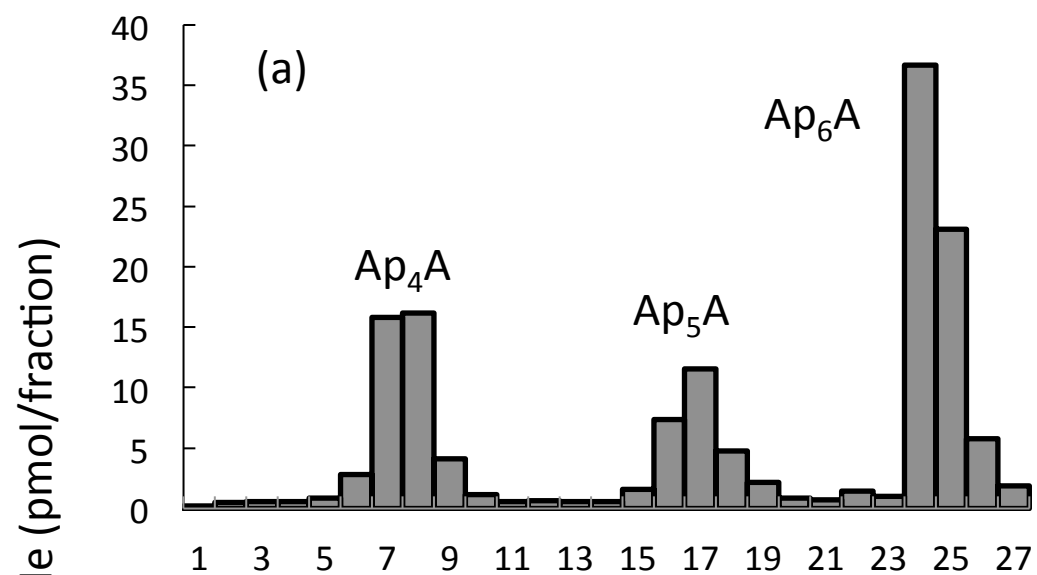




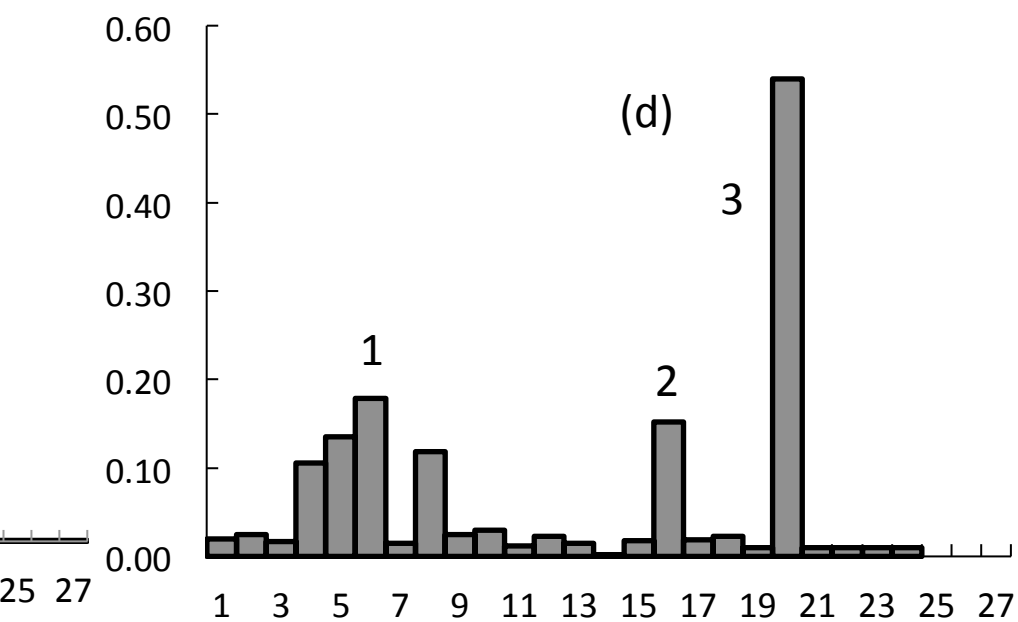
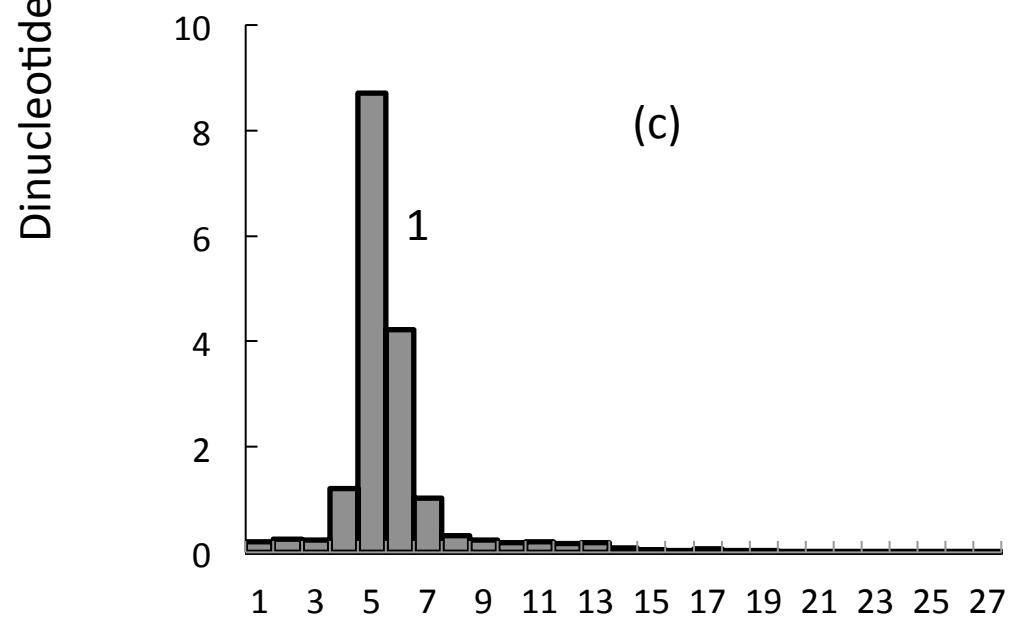
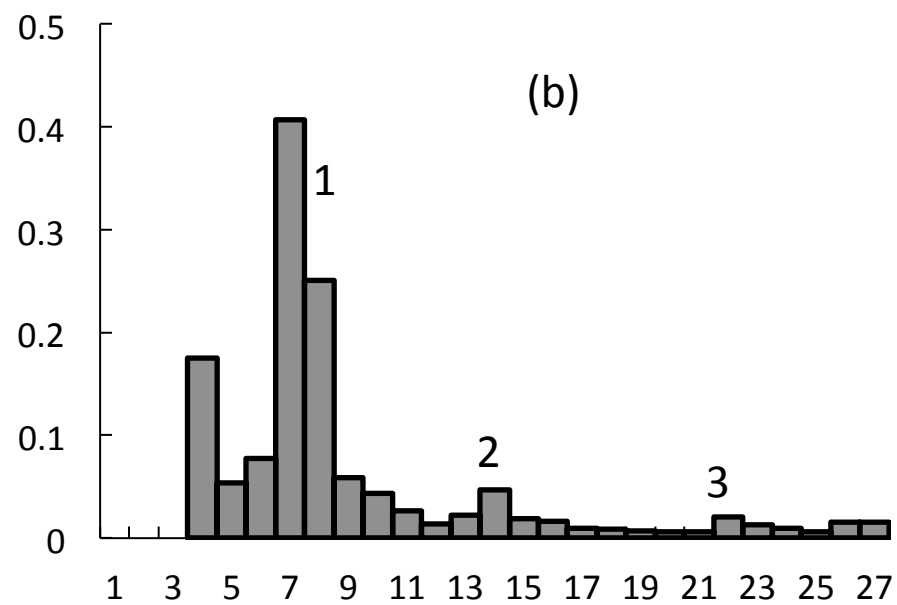
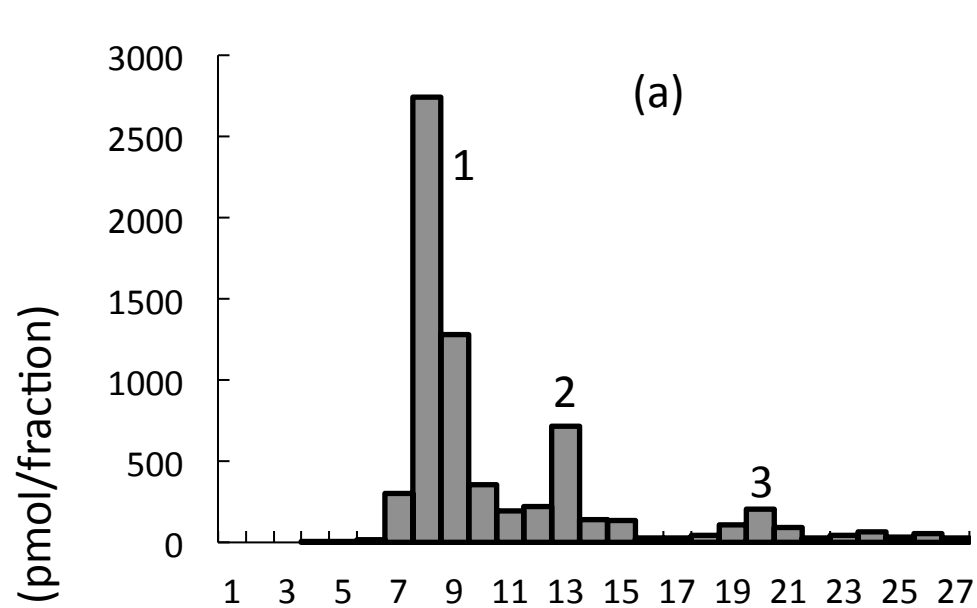






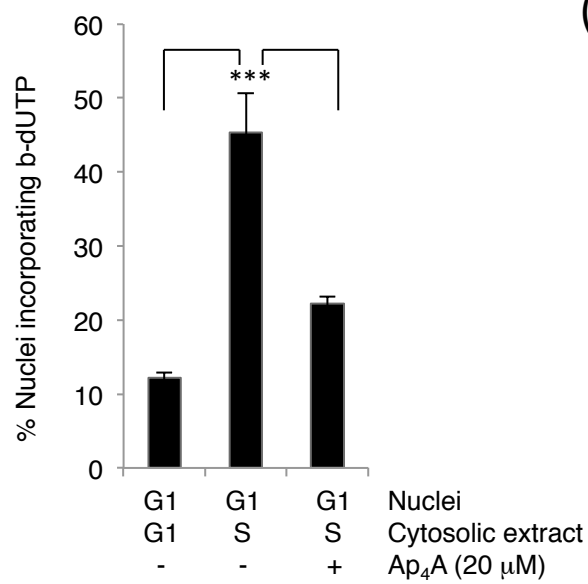


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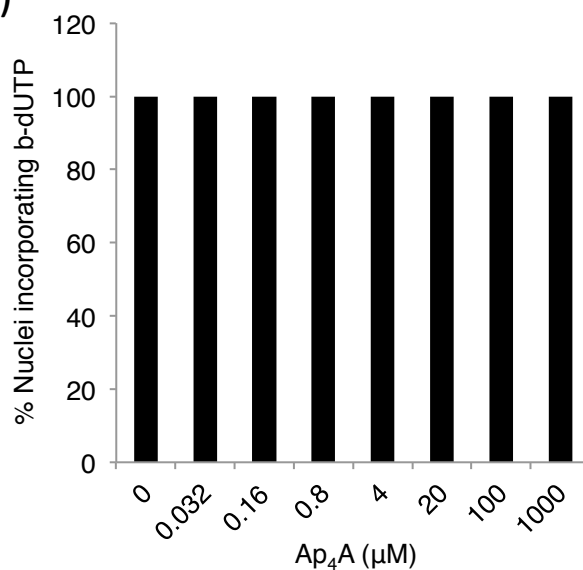


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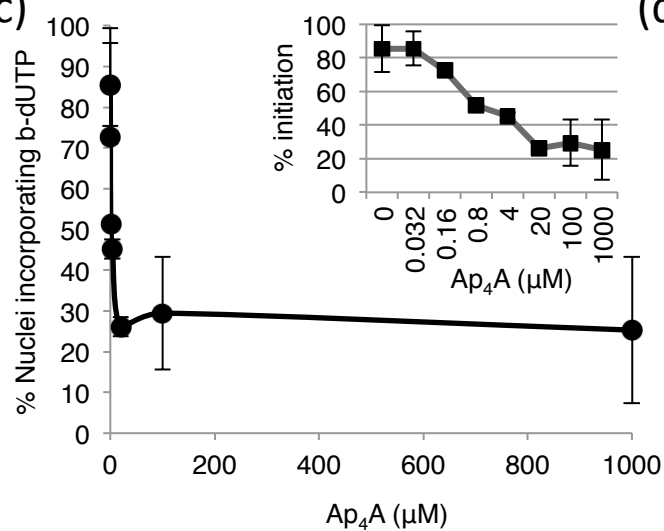
(a)



(b)



(c)



(d)

